STUDIES OF TWO KINDS OF VIRUS PARTICLES WHICH COMPRISE INFLUENZA A2 VIRUS STRAINS

II. Reactivity with Virus Inhibitors in Normal Sera*

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In previous communications (1, 2) separation and characterization of two kinds of particles with contrasting properties from influenza A2 virus strains were reported. Furthermore, it was demonstrated that variation in properties among the strains was due to fluctuation from strain to strain in the proportions of the two kinds of particles.

Particles characterized by high sensitivity to specific antibody and by high reactivity with mucoprotein receptors were designated "+", and particles with low sensitivity to antibody and low reactivity with receptors were called "−". Substrains of the "+" and "−" particles remained homogeneous and genetically stable on serial passage in the chicken embryo.

It was reported previously that horse serum in high dilution neutralized "+" particles, but had no effect on "−" particles (1, 2). Rabbit serum had a similar effect but was less active than horse serum. Both horse and rabbit sera also inhibited hemagglutination by "+", but not by "−" particles.

In the present communication hemagglutination-inhibiting and neutralizing activities of sera from seven animal species are reported. Some of the properties of normal serum components with hemagglutination-inhibiting and neutralizing activities for "+" particles are described. Human, rabbit, and horse sera were separated by starch zone electrophoresis, and it will be shown that all inhibitory components possessed both hemagglutination-inhibiting and neutralizing activities for "+" particles. The effects of various chemical and physical agents on the hemagglutination-inhibiting and neutralizing activities of whole sera and electrophoretically separated serum fractions are described.

The mechanism of interaction of "+" and "−" particles with mucoprotein receptors is discussed. The unusually high reactivity of "+" particles with such receptors is analyzed and possible reasons for the failure of "−" particles to react with inhibitors in normal animal sera are considered.

Materials and Methods

Viruses.—The pure substrains of influenza A2 virus ($RI/4^+$, $RI/4^-$, $RI/5^+$, and $RI/5^-$), the parent strain, RI/5, and the Lee strain of influenza B virus employed in this study were described in the preceding communication (2).

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Buffers.—The composition of phosphate buffered saline (PBS) and of calcium borate-buffered saline (CaBBS) were given in the preceding communication (2).

Sera.—Sera were obtained from normal chickens, ferrets, hamsters, and mice. Normal human sera were obtained prior to the influenza epidemic in New York in 1957, and no antibodies against influenza A2 virus isolates could be demonstrated in any of these sera in hemagglutination-inhibition tests after *Vibrio cholerae* filtrate treatment. Normal horse and rabbit sera and human convalescent sera were obtained, and all sera were prepared and stored as described in the preceding communication (2).

Purified Neuraminidase.—Except where otherwise stated, the preparation of purified neuraminidase described in the preceding communication (2) was employed.

V. cholerae Filtrate.—The preparation of *V. cholerae* filtrate and the procedure of treatment of sera were described in the preceding communication (2).

Trypsin Treatment of Sera.—Sera were treated with trypsin by the procedure of Jensen and coworkers (3). Solutions of crystalline trypsin (Worthington Biochemical Corporation) were prepared fresh in PBS to give 4 mg. of trypsin per ml. of serum in the final mixture of 4 parts of trypsin solution to 1 part of serum. Mixtures were incubated at 56°C. for 30 minutes.

Periodate Treatment of Sera.—The procedure used to treat sera with periodate was a modification of the method described by Granoff and Hirst (4). Four parts of serum were mixed with one part of sodium periodate solution to give a final concentration of periodate of 0.02 M. Mixtures were held at 24°C. for 30 minutes, and one part of 40 per cent glucose was added to neutralize the periodate.

Neutralization Tests.—The constant virus-serum dilution technique using a virus inoculum of 1000 EID_{50} was employed as described in the preceding communication (2).

Electrophoretic Methods.—Electrophoretic separation of sera was performed by the method of Kunkel (5), using starch as the supporting medium. Separation was carried out at 4°C. in barbital buffer at pH 8.6, ionic strength 0.1. The duration of electrophoresis was 21 to 24 hours, and a potential gradient of 8.9 volts/cm. was employed.

The starch block was cut into 1 centimeter sections and each section was eluted with 5 ml. of PBS containing 250 units of penicillin and 250 mg. of streptomycin per ml. The protein content of each fraction was assayed by a modified Folin procedure (6). Fractions were stored at −25°C. until used.

EXPERIMENTAL

Inhibitory Activity of Normal Animal Sera.—Information concerning the susceptibility of several influenza A2 virus strains to inhibition by normal human, horse, and rabbit serum in hemagglutination and neutralization reactions was reported previously from this laboratory (1, 7, 8). In a comparative study of inhibitory activities with sera from 7 animal species the RI/5 strain was used as the test virus.

The passage of RI/5 strain employed was composed predominantly of inhibitor-sensitive ("+") virus particles; the ratio of "+" to "−" particles was approximately 10,000:1 (2). In neutralization titrations, a constant virus inoculum of 1000 EID_{50} per egg and serial serum dilutions were used. The hemagglutination-inhibition tests were done by the usual procedure. All sera were heated at 56°C. for 30 minutes.

As can be seen in Table I, mouse and hamster sera showed slight hemagglutination-inhibiting activity; human, chicken, ferret, and rabbit sera showed moderate activity, and horse serum showed very high activity. The results of

neutralization titrations were similar to those obtained in hemagglutination-inhibition reactions. It should be emphasized that sera from 5 species, *i.e.* man, chicken, ferret, rabbit, and horse, caused neutralization as well as hemagglutination-inhibition, although the sera had been heated at 56°C. for 30 minutes.

Neutralization of the infectivity of some strains of influenza A2 virus by normal human and animal sera has been reported from several laboratories (8–13). Cohen and Belyavin (9) have reported high neutralizing activity in normal horse serum and moderate activity in rabbit serum, which agrees with the results described above. Levy and coworkers (10) also reported neutralization of some influenza A2 viruses by horse and rabbit sera, but, in contrast to the results with human serum reported above and previously (8, 11), no neutralization by normal human serum. However, failure of human serum to neutralize influenza A2 viruses (10) may have been due to the presence in the strains of too great a proportion of inhibitor-insensitive particles. Neutralization of influenza A2 virus strains by normal guinea pig serum has also been reported (11, 13).

TABLE I

Neutralization and Hemagglutination-Inhibiting Activities of Normal Sera with RI/5 Virus

Sera*		Geometric mean titer with RI/5 strain	
Species	No.	Neutralization‡	Hemagglutination-inhibition§
Mouse	20	<8	16
Hamster	4	<8	128
Man	6	17	512
Chicken	2	43	720
Ferret	2	44	1,450
Rabbit	6	52	1,450
Horse	4	166	23,400

* Heated at 56°C. for 30 minutes.
‡ Inoculum: 1000 EID_{50} per egg.
§ Amount of virus: 4 HA per tube.

Normal horse serum was also tested for hemagglutination-inhibiting activity against several influenza viruses isolated prior to 1957: an influenza A strain (PR8), two influenza A1 strains (FM1 and NA), and an influenza B strain (Lee, heated at 56°C. and unheated). The horse serum was used without heating and after heating at 56°C. for 30 minutes. There was no inhibition of PR8 or NA viruses. With FM1 virus very low titers were obtained with both unheated and heated serum. With both heated and unheated Lee virus there was no inhibition by heated serum, and only very slight inhibition by unheated serum. These results are in marked contrast to those obtained above with the inhibitor-sensitive RI/5 strain of influenza A2 virus. Cohen and Belyavin found only one virus isolated prior to 1957 which was highly sensitive to horse serum (9).

Elimination of Inhibitory Activity of Normal Sera.—The ability of viral en-

zyme, *V. cholerae* filtrate, purified neuraminidase, trypsin, and sodium periodate to eliminate the hemagglutination-inhibiting activity of normal animal sera for "+" particles was investigated.

All sera were initially heated at 56°C. for 30 minutes. As will be described below, heating causes changes in inhibitory activity of some sera. Therefore, since some of the methods of treatment require different amounts of heat, each was individually controlled. Controls consisted of serum and the appropriate diluent for the experimental reagent; normal allantoic fluid in the case of viral enzyme, neopeptone broth for *V. cholerae* filtrate, and PBS for the other experimental reagents.

TABLE II

Effect of "+" and "−" Virus Particles, Neuraminidase, V. cholerae Filtrate, Trypsin, and Sodium Periodate on Hemagglutination-Inhibiting Activity of Normal Sera

Treatment of serum*	Geometric mean hemagglutination-inhibition titer‡			
	Human	Ferret	Rabbit	Horse
Control	1,950	3,280	1,070	66,600
RI/5$^+$	1,480	2,560	443	32,300
Control	2,180	3,280	810	53,400
RI/5$^-$	2,180	3,280	722	53,400
Control	2,048	4,096	715	40,600
Neuraminidase	64	32	64	27,400
Control	2,048	4,096	2,048	67,500
V. cholerae filtrate	<16	16	91	37,300
Control	2,560	3,260	1,280	81,920
Trypsin, 4 mg./ml.	113	160	160	1,810
Control	1,556	3,120	1,450	92,600
0.02 M $NaIO_4$	<12	<12	<12	<12

* Initially all samples heated at 56°C. for 30 minutes.

‡ Amount of virus: 4 HA units of RI/4$^+$ per tube.

The RI/5$^+$ and RI/5$^-$ substrains were used as viral enzyme preparations. Mixtures of 0.3 ml. of serum and 2.7 ml. of infected allantoic fluid containing approximately 2800 HA units of virus were incubated in a water bath for 3 hours at 37°C. The mixtures were then heated at 65°C. for 30 minutes to destroy the hemagglutinating activity of the virus, and the hemagglutination-inhibition titer determined. The treatment with purified neuraminidase was carried out by mixing 0.3 ml. volumes of serum with equal volumes of a neuraminidase solution containing 200 units of enzyme per ml (14). Mixtures were incubated at 37°C. for 3 hours and then heated at 56°C. for 30 minutes to inactivate the enzyme.

The remaining inhibitory activity after treatment was assayed in hemagglutination-inhibition titrations with 4 HA units of the RI/4$^+$ substrain. All titers are expressed as the reciprocal of the final dilution of serum at end point.

Table II summarizes the results of these experiments. The RI/5$^+$ substrain caused some reduction in inhibitory activity of all of the sera tested. In contrast the RI/5$^-$ substrain caused no significant reduction in titers of any of the sera. These results are consistent with the previous finding that the RI/5$^-$ particles were not inhibited by these sera. It is shown below that "−" particles do not combine with inhibitory components in horse serum. Failure of RI/5$^-$ particles to alter enzymatically the virus inhibitory components in sera from several animal species supports the view that "−" particles do not combine with these components.

Purified neuraminidase and *V. cholerae* filtrate caused a marked reduction in the inhibitory activity of human, ferret, and rabbit sera. The hemagglutination-inhibiting activity of horse serum was reduced to a moderate extent by RI/5$^+$ viral enzyme (51 per cent), purified neuraminidase (33 per cent), and *V. cholerae* filtrate (45 per cent). The values shown in Table II represent the geometric mean values based on a large number of experiments. The data were examined in the t test using the method of paired comparisons. The number of experiments with each reagent and the p values obtained were as follows: *V. cholerae* filtrate, 14 experiments, $p = <0.001$; RI/5$^+$ viral enzyme, 10 experiments, $p = <0.01$; purified neuraminidase, 11 experiments, $p = <0.01$. Thus, the reduction in virus inhibitory activity of horse serum caused by these agents is significant.

The failure of these three reagents to eliminate completely the inhibitory activity of horse serum was not due to an insufficient quantity of enzyme. When fivefold greater amounts of purified neuraminidase or *V. cholerae* filtrate and a 1:200 dilution of horse serum instead of undiluted serum were used, a similar result was obtained; *i.e.*, approximately 50 per cent reduction in inhibitory activity.

The time course of inactivation of horse serum inhibitors was also investigated.

As substrate, undiluted and a 1:100 diluted normal horse serum were used. Treatment with *V. cholerae* filtrate and neuraminidase was carried out as described above except that aliquots of serum were treated for varying periods of time. The purified neuraminidase was obtained from Dr. A. Rosenberg of Columbia University. Appropriate controls were included for each time period. The inhibitory activity of the controls remained unchanged throughout the course of the experiments.

As shown in Fig. 1, the maximum reduction caused in hemagglutination-inhibiting activity of normal horse serum by both *V. cholerae* filtrate and purified neuraminidase was 50 per cent, though treatment was carried out for as long as 18 hours. As would be expected, when diluted serum was used the maximum reduction was achieved sooner than with undiluted serum. These results are in agreement with those obtained above, and indicate that approximately 50 per cent of the inhibitory activity of normal horse serum is not affected by neuraminidase or *V. cholerae* filtrate.

Trypsin caused a considerable reduction in the virus inhibitory activity of all sera (Table II), but did not completely eliminate such activity. Only sodium periodate caused complete elimination of hemagglutination-inhibiting activity of all sera.

The failure of the receptor-destroying enzyme (RDE) of *V. cholerae* and of trypsin to eliminate completely from some sera the hemagglutination-inhibiting activity for inhibitor-sensitive influenza A2 virus strains was recognized early in the course of the 1957 pandemic (15). Cohen and Belyavin (9) found that trypsin reduced the inhibitory titer of normal horse serum significantly, but the hemagglutination-inhibition titer of serum was >10,240 both before and after treatment of serum by RDE. However, partial inactivation of the degree described above would not have been detected in such titrations.

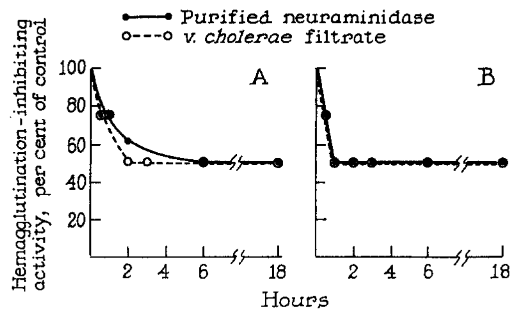

FIG. 1. Time course of inactivation of inhibitory activity of normal horse serum by purified neuraminidase and *V. cholerae* filtrate. A, undiluted horse serum; B, horse serum diluted 1:100. Serum was heated at 56°C. for 30 minutes prior to treatment. The temperature of the reaction mixtures was 37°C. Residual inhibitory activity was determined with the RI/4$^+$ substrain.

The complete removal of inhibitory activity by 0.011 M potassium periodate has been reported (16). The results obtained in the present study are in agreement with the earlier findings.

The effects of *V. cholerae* filtrate, trypsin, and sodium periodate treatment on the neutralizing activity of normal rabbit serum for RI/5 strain were also determined. Results were similar to those obtained above in hemagglutination-inhibition titrations. *V. cholerae* filtrate and trypsin caused a 42 and 64 per cent reduction, respectively, in inhibitory activity, but only sodium periodate caused a complete elimination of activity.

Combination of Virus and Horse Serum Inhibitors.—As described in the preceding communication (2), only the "+" virus particles were sensitive to serum inhibitors, whereas the "−" virus particles were completely insensitive. Neutralization of "+" virus particles by serum inhibitors suggests that a stable union is formed between virus and inhibitor. Combination of virus and inhibitors was investigated in experiments in which virus-serum mixtures were subjected to high speed centrifugation, and the amount of inhibitor that sedimented with the virus was determined.

One ml. volumes of a 1:10 dilution of normal horse serum in PBS were added to 14 ml. volumes of infected allantoic fluid containing 1024 HA units per ml. of either "+" or "−" particles or to 14 ml. of PBS as a control. The mixtures were held at 24°C. for 1 hour and at 4°C. overnight. The mixtures were then centrifuged in the Spinco Model L centrifuge at 27,000 *g* for 1 hour. Such centrifugation sediments the virus, but will not sediment uncombined serum inhibitors. The upper one-fourth of the supernates were tested for hemagglutination-inhibiting activity with 4 HA units of the inhibitor-sensitive substrain $RI/4^+$. Titers are expressed as the reciprocal of the final dilution of serum at end point.

In five such experiments, horse serum inhibitors sedimented with the "+" substrains but not the "−" substrains. Table III shows the results of a typical

TABLE III

Adsorption of Horse Serum Inhibitors by Virus

Adsorbing virus	Serum hemagglutination-inhibition titer* after removal of virus by centrifugation
None	4800‡
$RI/4^+$	600
$RI/4^-$	4800
$RI/5^+$	600
$RI/5^-$	4800

* Amount of virus: 4 HA units of $RI/4^+$ per tube.

‡ Before centrifugation the titer was also 4800.

experiment. A large amount of the serum inhibitor was sedimented with both the $RI/4^+$ and $RI/5^+$ particles, whereas none was sedimented with the $RI/4^-$ and $RI/5^-$ particles. The "+" particles are thus able to form a complex with horse serum inhibitor molecules, but the "−" particles are either unable to combine with inhibitor or rapidly dissociate from it.

Effect of Heat on Inhibitory Activity of Normal Horse Serum.—As was shown above, the neutralizing activity of inhibitors in horse serum was not eliminated by heating the serum at 56°C. for 30 minutes. The effect of heating normal horse serum at various temperatures on the hemagglutination-inhibiting and neutralizing activities of serum were determined.

Undiluted normal horse serum was heated for 30 minutes or one hour at varying temperatures. Before serum was heated at 65°C., it was diluted 1:2 with PBS. After heating, the inhibitory activity of the serum was determined using the RI/5 strain of influenza A2 virus.

As can be seen in Table IV, both hemagglutination-inhibition and neutralization titers of normal horse serum increased after heating. The titers found after heating at 65°C. were extraordinarily high.

The inhibitory activity of heated horse serum was not reduced when unheated

horse serum was added. This indicates that the increase in virus inhibitory activity on heating was not due to elimination of an agent capable of blocking the action of serum inhibitors. The increase in inhibitory activity that is produced when horse serum is heated suggests an unfolding or a disaggregation of the native protein with "unmasking" of additional virus receptor sites.

Using an inhibitor-sensitive influenza A2 strain, Harboe (17) found an increase in hemagglutination-inhibiting activity of the sera of several species after heating at 62°C. for 30 minutes, and Takátsy and Barb (11) also found an increase in hemagglutination-inhibiting activity after boiling sera for 30 minutes. However, horse serum was not studied by these authors.

TABLE IV

Increase in Neutralizing and Hemagglutination-Inhibiting Activities of Normal Horse Serum with Heating

Treatment of serum	Serum titer with RI/5 virus	
	Neutralization*	Hemagglutination-inhibition‡
None	128	10,240
37°C. 30 min.	137	10,240
56°C. 30 "	297	20,480
65°C. 30 "	2,740	163,800
65°C. 60 "	3,410	327,700

* Inoculum: 1000 EID_{50} per egg.
‡ Amount of virus: 4 HA units per tube.

Effect of Urea on Inhibitory Activity of Normal Horse Serum.—Because heat caused a marked increase in inhibitory activity of horse serum, the effect of urea, another agent which causes denaturation of proteins, was investigated.

One or 1.5 ml. volumes of unheated normal horse serum were mixed with concentrated urea solutions to give final urea concentrations of 7, 8, or 9.3 M. Controls consisted of normal horse serum mixed with PBS. Mixtures were held at 4°C. for 16 to 24 hours and then dialyzed for 54 hours at 4°C. against 350 volumes of PBS. After dialysis the hemagglutination-inhibiting activity of all samples was determined with 4 HA units of the $RI/4^{+}$ substrain. The inhibitory titer was expressed as the reciprocal of the final dilution of serum at the end point.

Table V shows that treatment with urea caused an 8- to 32-fold increase in the inhibitory activity of horse serum. The greatest increase was observed after treatment of serum with 8 M urea for 24 hours. These results are consistent with the above suggestion that the increase in inhibitory activity is due to "unmasking" of virus receptor sites by unfolding of the native protein molecule.

Effect of Freezing on Inhibitory Activity of Normal Horse Serum.—Freezing and thawing of normal horse serum from one to five times has no effect on the hemagglutination-inhibiting activity of serum as determined with the RI/5 strain.

Electrophoresis of Normal Horse Serum.—Normal horse serum was subjected to starch zone electrophoresis, and the hemagglutination-inhibiting and neutralizing activities of the separated fractions were determined.

Serum was heated at 56°C. for 30 minutes prior to electrophoresis. The RI/5 strain of virus was used to assay inhibitory activity.

Fig. 2 shows the inhibitory activity of the electrophoretic fractions of normal horse serum. The high inhibitory activity of some of the fractions reflects the high level of activity found in whole serum. The curves depicting the hemagglutination-inhibiting and neutralizing activities of the electrophoretic fractions

TABLE V

Increase in Hemagglutination-Inhibiting Activity of Normal Horse Serum by Treatment with Urea

Concentration of urea	Serum hemagglutination-inhibition titer*	
	Duration of treatment	
	16 hrs.	24 hrs.
None	6,400	6,400
7 M	51,200	51,200
None	4,800	4,800
8 M	76,800	153,600
None	7,200	3,600
9.3 M	57,600	57,600

* Amount of virus: 4 HA units of $RI/4^{+}$ per tube.

of serum are closely similar. This suggests that the same serum components possess both activities.

As can also be seen in Fig. 2, there are two major peaks of inhibitory activity. The highest peak was found with the major α-globulin component, probably migrating with the α2-globulin, and the second peak was found in the albumin regions. In the pattern shown in Fig. 2, the second peak was found in the slow albumin region; however, on electrophoresis of other sera it has also been found in the center or the fast albumin regions. A shoulder of low hemagglutination-inhibiting and neutralizing activity can be seen in the β-globulin region. No inhibitory activity was found in the γ-globulin region.

Levy and coworkers (10) have reported that hemagglutination-inhibiting activity for an inhibitor-sensitive strain of influenza A2 virus migrated in two components in horse serum: in the α1-globulin and in the slow albumin region. Hamburg (18) found inhibitory activity in the α1, α2, and sometimes β-globulin fractions of horse serum.

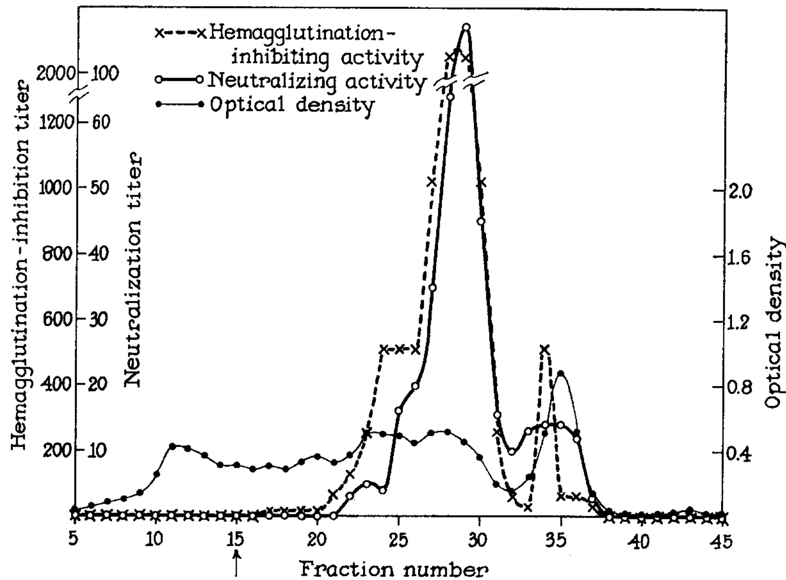

FIG. 2. Hemagglutination-inhibiting and neutralizing activities of electrophoretically separated fractions of normal horse serum. Serum was heated at 56°C. for 30 minutes prior to electrophoresis. Inhibitory activity was determined with the RI/5 strain.

Effect of Heat on Inhibitory Activity of Electrophoretically Separated Fractions of Normal Horse Serum.—Electrophoretic separation of horse serum made it possible to determine whether the marked increase in hemagglutination-inhibiting and neutralizing activities produced by heating of whole serum was due to an increase in the activities of only one or of all of the components in unheated serum which showed activity.

Normal horse serum was subjected to starch zone electrophoresis without prior treatment. The hemagglutination-inhibiting and neutralizing activities of serum fractions were then determined without heating and also after heating at 65°C for 30 minutes. The RI/5 strain of virus was employed in the titrations.

Fig. 3 depicts the hemagglutination-inhibiting activity of electrophoretic fractions. It can be seen that heating markedly increased the inhibitory activity present in two peaks in the α-globulin and albumin regions. The small amount of activity present in the β-globulin region increased also.

In this experiment inhibitory activity was found in essentially the same regions as in the experiment shown in Fig. 2 in which horse serum was heated at 56°C. prior to electrophoresis.

Fig. 4 describes the neutralizing activity of heated and unheated serum fractions. It can be seen that heating markedly increased the neutralizing activity in the two peaks in the α-globulin and albumin regions. The low

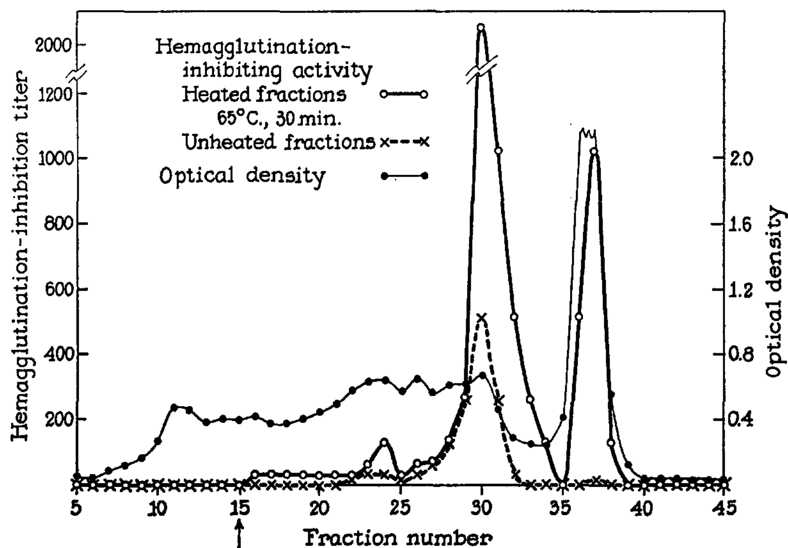

FIG. 3. Effect of heat on the hemagglutination-inhibiting activity of electrophoretically separated fractions of normal horse serum. Fractions were tested for inhibitory activity unheated and after heating at 65°C. for 30 minutes. Inhibitory activity was determined with the RI/5 strain.

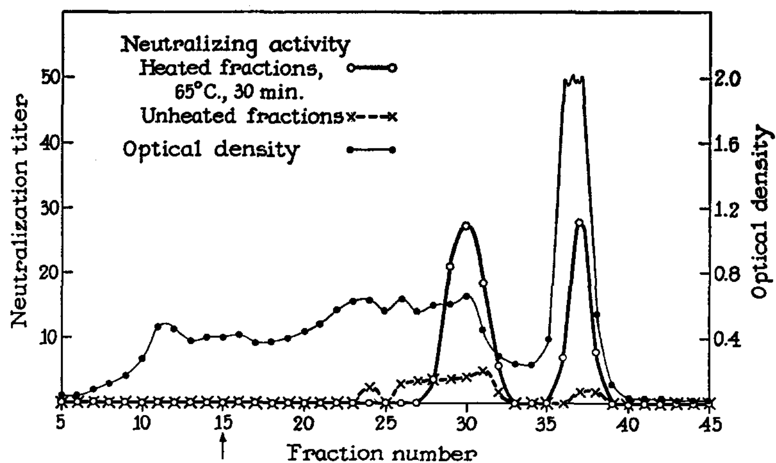

FIG. 4. Effect of heat on the neutralizing activity of electrophoretically separated fractions of normal horse serum. Fractions were tested for neutralizing activity unheated and after heating at 65°C. for 30 minutes. Neutralizing activity was determined with the RI/5 strain.

neutralizing activity of the unheated fractions in the β-globulin region disappeared after heating.

In summary, the hemagglutination-inhibiting and neutralizing activity

of the two major inhibitory components in normal horse serum increased in parallel on heating.

Effect of V. cholerae Filtrate on Inhibitory Activity of Fractions of Normal Horse Serum.—The finding described above that viral enzyme, purified neuraminidase, and *V. cholerae* filtrate all reduced the hemagglutination-inhibiting activity of normal horse serum by 33 to 51 per cent, raised the possibility that one of the two major peaks of activity found on electrophoresis might be affected and not the other. To investigate this possibility the electrophoretically

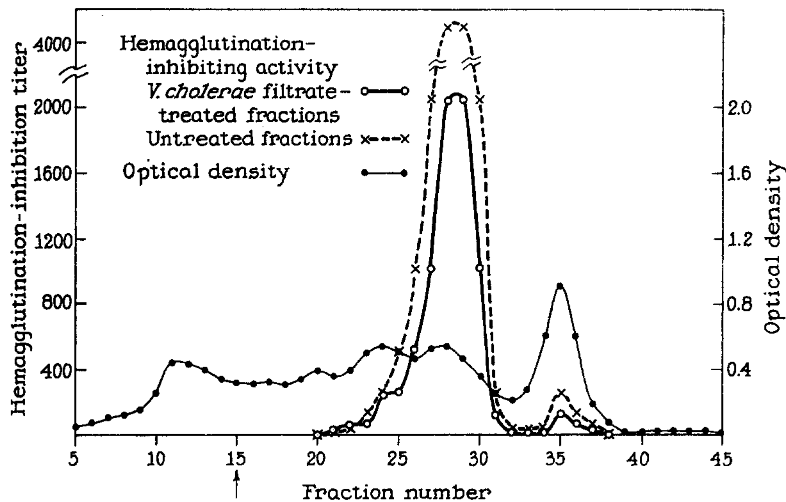

FIG. 5. Effect of *V. cholerae* filtrate on the hemagglutination-inhibiting activity of electrophoretically separated fractions of normal horse serum. Serum was heated at 56°C. for 30 minutes before electrophoresis. Inhibitory activity was determined with the RI/5 strain.

separated fractions of normal horse serum were treated with *V. cholerae* filtrate and their hemagglutination-inhibiting activity compared with the activity of untreated controls. The horse serum was heated at 56°C. for 30 minutes prior to electrophoresis.

As can be seen in Fig. 5, treatment with *V. cholerae* filtrate caused a 50 per cent decrease in inhibitory activity in almost every fraction, and the major peaks were reduced to a similar degree. It may be concluded that the decrease in inhibitory activity of whole serum caused by treatment with *V. cholerae* filtrate and the other agents with receptor-destroying enzymatic activity is not due to an effect on only one of the serum components with inhibitory activity. Treatment of fractions in α-globulin region with purified neuraminidase also caused a 50 per cent decrease in inhibitory activity. Other fractions were not tested.

Electrophoresis of Normal Human and Rabbit Sera.—Normal human and

rabbit sera were subjected to starch zone electrophoresis, and the hemagglutination-inhibiting and neutralizing activities of the separated fractions were determined.

Sera were heated at 56°C. for 30 minutes prior to electrophoresis. The RI/5 strain of virus was used to assay inhibitory activity.

Fig. 6 shows the inhibitory activity of the electrophoretically separated fractions of normal human serum. As in the case of horse serum, the curves representing hemagglutination-inhibiting and neutralizing activities are closely

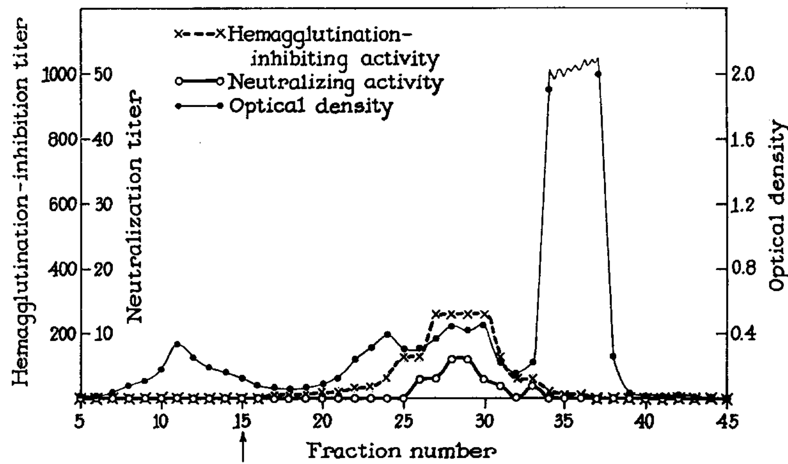

FIG. 6. Hemagglutination-inhibiting and neutralizing activities of electrophoretically separated fractions of normal human serum. Serum was heated at 56°C. for 30 minutes prior to electrophoresis. Inhibitory activity was determined with the RI/5 strain.

similar, suggesting that the two activities are associated with the same serum components. There is a broad peak of inhibitory activity with the major α-globulin component which probably migrated with the α2-globulins, and a small shoulder of activity anterior to this which probably migrated with the α1-globulins. No activity was found in the γ-globulin region.

Levy *et al.* (10) found hemagglutination-inhibiting activity for an inhibitor-sensitive influenza A2 strain in the α2- and α1-globulin regions of human serum.

Fig. 7 shows the inhibitory activity of the electrophoretic fractions of rabbit serum. Again, the curves representing the hemagglutination-inhibiting and neutralizing activities are very similar. The major portion of activity was found in a broad peak in the α-globulin region, and there was a shoulder in the β-globulin region. In no instance has inhibitory activity been found in the

γ-globulin region in normal horse, human, or rabbit serum, where activity should be found if it were due to specific antibody.

Levy and coworkers (10) found hemagglutination-inhibiting activity in the β- and α1-globulin regions of normal rabbit serum. The slight differences between the results reported earlier and those shown in Fig. 7 are probably due to the heating of the serum at 56°C. prior to electrophoresis in the latter instance. In experiments to be described below (Fig. 6) serum was subjected to electrophoresis without prior heating and these results correspond more closely to those of Levy and coworkers (10). Using the A/Norway/9, 57 strain of influenza A2 virus, Harboe (17) found hemagglutination-inhibiting activity in all fractions of rabbit serum which contained α- or β-globulins.

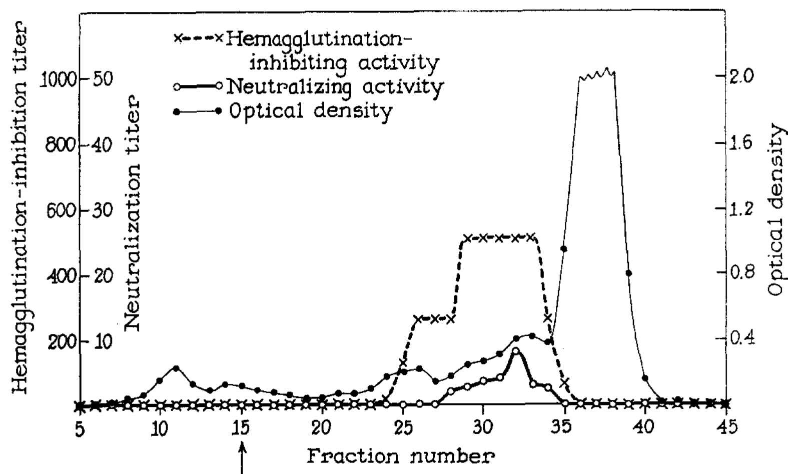

FIG. 7. Hemagglutination-inhibiting and neutralizing activities of electrophoretically separated fractions of normal rabbit serum. Serum was heated at 56°C. prior to electrophoresis. Inhibitory activity was determined with the RI/5 strain.

Effect of Heat on Inhibitory Activity of Normal Rabbit Serum.—The effect of heat on the hemagglutination-inhibiting and neutralizing activities of normal rabbit serum was investigated.

Aliquots of undiluted serum were heated at various temperatures for 30 minutes or 1 hour. Before heating at 65°C. serum was diluted 1:2 with PBS. The RI/5 strain was used to assay inhibitory activity.

As can be seen in Table VI, heating had no effect on the hemagglutination-inhibiting activity of rabbit serum; however neutralizing activity decreased on heating. In other experiments of this type similar results were obtained except that in a few experiments a 2-fold increase was observed in hemagglutination-inhibiting activity after heating at 65°C. for 30 minutes. In no experiment was neutralizing activity eliminated by heating, but a reduction in such

activity was regularly observed. Heating at 75°C. caused no greater decrease in neutralizing activity than heating at 65°C. These findings suggest that at least one of the components of rabbit serum which has inhibitory activity is sensitive to heat in so far as the ability to neutralize infectivity is concerned.

Harboe (17), using an inhibitor-sensitive influenza A2 strain, found no difference in hemagglutination-inhibition titers of unheated rabbit serum and rabbit serum heated at 62°C. for 30 minutes. Takátsy and Barb (11) reported a 1.6 to 3-fold increase in the hemagglutination-inhibition titer after boiling of normal rabbit serum.

TABLE VI

Effect of Heat on Neutralizing and Hemagglutination-Inhibiting Activities of Normal Rabbit Sera

Treatment of serum	Serum titer with RI/5 virus	
	Neutralization*	Hemagglutination-inhibition‡
None	64	1280
37°C. 30 min.	53	1280
56°C. 30 "	48	1280
65°C. 30 "	28	1280
65°C. 60 "	20	1280

* Inoculum: 1000 EID_{50} per egg.
‡ Amount of virus: 4 HA units per tube.

Effect of Heat on Inhibitory Activity of Electrophoretically Separated Fractions of Normal Rabbit Serum.—Because of the results obtained with heated whole rabbit serum, the electrophoretic fractions of rabbit serum were examined to determine the effect of heating on the hemagglutination-inhibiting and neutralizing activities of serum components.

Normal rabbit serum was subjected to starch zone electrophoresis without prior heating. The hemagglutination-inhibiting and neutralizing activities of each fraction were determined before heating and after heating the fraction at 65°C. for 30 minutes. The $RI/5^+$ substrain was used to assay inhibitory activity.

The results of the hemagglutination-inhibition titrations with unheated and heated fractions are shown in Fig. 8. There was an apparent increase in activity of several fractions after heating and a decrease in two fractions; however, with one exception the changes in titers are only 2-fold and therefore their significance is questionable. The exception was that in fraction 33 a 4-fold increase was observed after heating. These results explain why heating of whole rabbit serum resulted in no detectable change or an occasional slight increase in hemagglutination-inhibition titers.

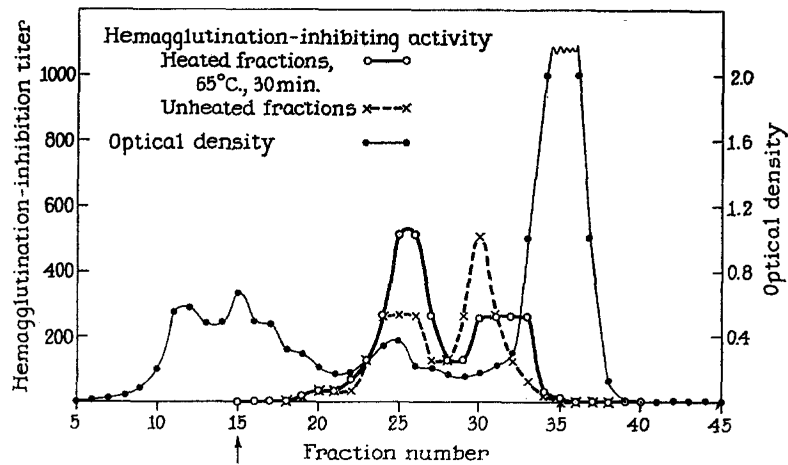

FIG. 8. Effect of heat on the hemagglutination-inhibiting activity of electrophoretically separated fractions of normal rabbit serum. Fractions were tested for inhibitory activity unheated and after heating at 65°C. for 30 minutes. Inhibitory activity was determined with RI/5+ substrain.

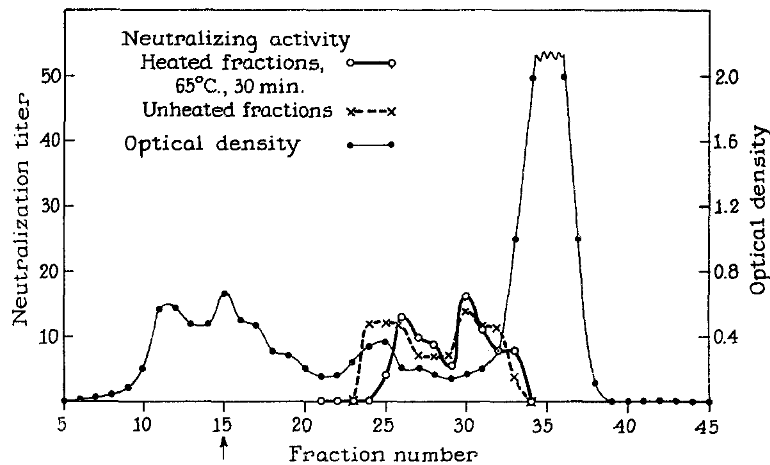

FIG. 9. Effect of heat on the neutralizing activity of electrophoretically separated fractions of normal rabbit serum. Fractions were tested for inhibitory activity unheated and after heating at 65°C. for 30 minutes. Inhibitory activity was determined with the RI/5+ substrain.

The results of neutralization titrations with heated and unheated fractions of rabbit serum are shown in Fig. 9. In the β-globulin region, there was a decrease in neutralizing activity after heating, but in the α-globulin region there were no significant changes in titer after heating.

Thus, there are inhibitory substances in normal rabbit serum which are capable of inhibiting hemagglutination and neutralizing infectivity of the inhibitor-sensitive influenza A2 virus particle. The ability of the inhibitor which migrated in the β-globulin region to neutralize infectivity was decreased by heat, in contrast to that of the inhibitor found in the α-globulin region, which was not significantly affected. These results with serum fractions explain the decrease in neutralizing activity of whole normal rabbit serum after heating.

It is of interest that neutralizing activity associated with β-globulin in normal horse serum also was reduced by heating, whereas that associated with α-globulin and albumin was increased, as described above.

Effect of Heat on the Inhibitory Activity of Human, Chicken, and Ferret Sera.—The effect of heat on the hemagglutination-inhibiting activity of human, chicken, and ferret sera was also investigated in titrations with the RI/5 strain. Heating at 56°C. for 30 minutes caused an increase in the inhibitory activity of each of these sera as compared to the activity of the unheated sera.

Harboe (17) found an increase in hemagglutination-inhibition activity of fowl, ferret, and guinea pig sera after heating at 62°C. for 30 minutes. Takátsy and Barb (11) found an increase in the hemagglutination-inhibiting activity of human, chicken, ferret, and guinea pig sera after boiling the serum for 30 minutes.

Effect of V. cholerae Filtrate on Inhibitory Activity of Fractions of Normal Rabbit Serum.—As shown above in Table II, purified neuraminidase and *V. cholerae* filtrate caused a marked reduction but not a complete elimination of inhibitory activity of rabbit serum. The effect of *V. cholerae* filtrate on the inhibitory activity of electrophoretically separated fractions of rabbit serum was investigated.

Serum was not heated prior to electrophoresis. The hemagglutination-inhibiting activity of electrophoretic fractions was determined with $RI/5^+$ substrain and compared to that of untreated controls.

As shown in Fig. 10, the inhibitory activity of all fractions in both the α- and β-globulin regions was reduced by *V. cholerae* filtrate to below the lowest level detectable in the titrations; *i.e.*, to a titer of <16. This finding does not necessarily indicate that inhibitory activity was completely eliminated. Furthermore, since the hemagglutination-inhibition titer after treatment of whole serum with *V. cholerae* filtrate was relatively low, 91, the failure to find a titer of 16 or higher in any of the fractions is not unexpected. Therefore, the serum component responsible for the activity remaining after treatment of whole serum with *V. cholerae* filtrate could not be identified.

Comparison of the Electrophoretic Mobilities of Inhibitors of RI/5 and Lee Viruses.—Electrophoretic fractions of normal human and rabbit serum were tested for hemagglutination-inhibiting activity to determine whether the

inhibitory activity against RI/5 and Lee (heated) viruses was associated with the same serum component.

Normal human and rabbit sera were subjected to starch zone electrophoresis. Human serum was heated at 56°C. for 30 minutes prior to electrophoresis. Rabbit serum fractions were heated at 65°C. for 30 minutes after electrophoresis. The Lee strain was heated at 56°C. for 30 minutes before use in hemagglutination-inhibition titrations.

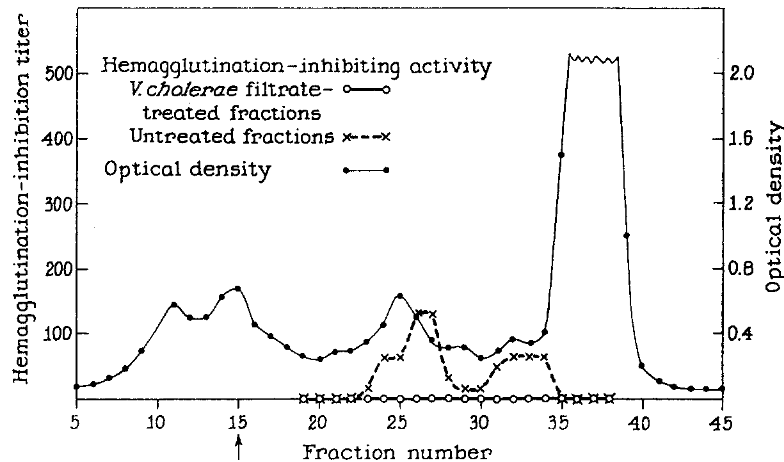

FIG. 10. Effect of *V. cholerae* filtrate on hemagglutination-inhibiting activity of electrophoretically separated fractions of normal rabbit serum. Inhibitory activity was determined with the RI/5+ substrain.

The results of these experiments are recorded in Fig. 11. As can be seen in part A of Fig. 11, the curve indicating inhibitory activity of human serum fractions against heated Lee is very similar to that of RI/5 virus. With rabbit serum the two curves have an almost identical shape, but the inhibition titers obtained with the heated Lee virus were lower than those determined with RI/5 virus.

It is of considerable interest that inhibitors for the new RI/5 virus and for heated Lee virus have the same electrophoretic mobility because the latter is widely known as the classical indicator of the Francis or α-inhibitor (19). This result parallels the findings described in the preceding communication (2) that inhibitor-sensitive influenza A2 virus particles are highly sensitive to urinary mucoprotein to which heated Lee virus is also highly sensitive.

Levy and coworkers (10) have reported inhibitory activity for heated Lee virus in the α2-globulin region of human serum. Inhibitory activity for an influenza A2 strain was found in the α2- and α1-globulin regions. These authors also reported that the inhibitory activity for heated Lee and the influenza A2 strain was found in the same fraction of normal rabbit

serum. Harboe also (17) found the inhibitory activity for heated Lee and for A/Norway/9/57 in the same electrophoretic fractions of rabbit serum; *i.e.*, those containing α- and β-globulins.

The above results with serum inhibitors and those with urinary mucoprotein described in the preceding communication (2) indicate that inhibitor-sensitive

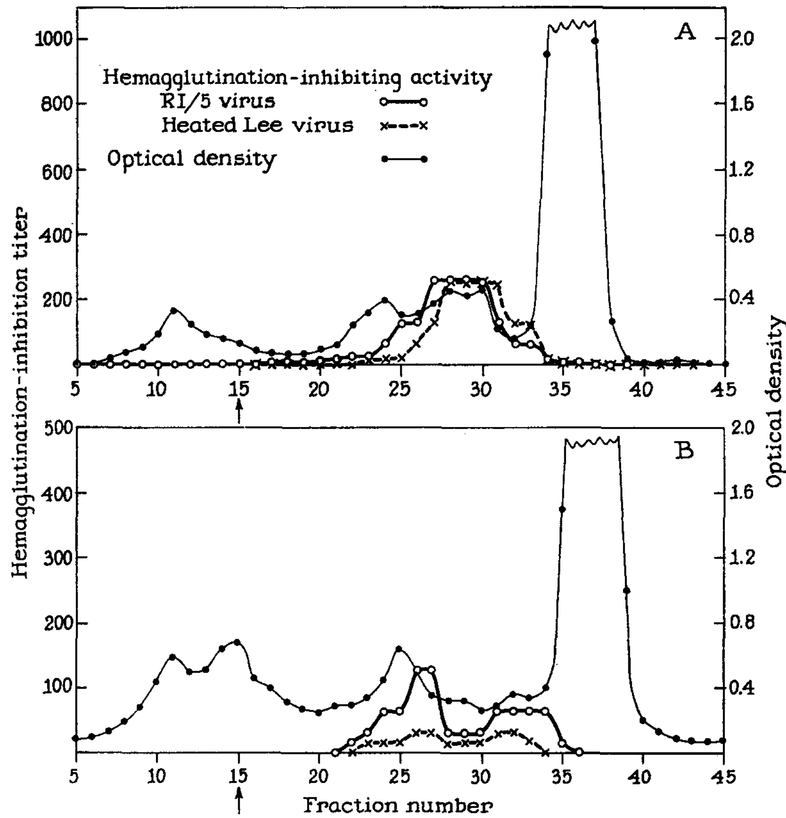

FIG. 11. Hemagglutination-inhibiting activity of electrophoretically separated fractions of normal human (A) and rabbit (B) sera with RI/5 and heated Lee virus. Human serum was heated at 56°C. for 30 minutes prior to electrophoresis.

A2 influenza virus particles are capable of combining with inhibitors in normal horse serum with which most of the pre-1957 influenza viruses cannot combine, but in addition they show that inhibitor-sensitive influenza A2 viruses can combine with urinary mucoprotein and with inhibitors in human and animal sera with which the earlier influenza viruses can also combine. Although the latter inhibitors prevent hemagglutination by both influenza A2 and pre-1957 influenza viruses, only the A2 viruses are neutralized by these heat-stable

serum inhibitors. This indicates a more stable bond between the inhibitor-sensitive A2 virus and the inhibitors.

DISCUSSION

Results of studies of normal animal and human serum components which possess hemagglutination-inhibiting and neutralizing activities for "+" but not for "−" particles have significantly contributed to the understanding of the mechanism of the unusual reactivity characteristics exhibited by these particles. The pertinent evidence will be discussed in two parts, the first dealing with studies with horse serum and the second with results obtained with rabbit and other sera. Reference is also made to findings reported in the preceding communication.

Unique Sensitivity of "+" Particles to Inhibitors in Normal Horse Serum.—It should be re-emphasized that in both hemagglutination-inhibition and neutralization reactions, "+" particles are extraordinarily sensitive to horse serum, and that "−" particles are completely insensitive. Indeed, it has been shown that "−" particles fail to combine with the inhibitory components in horse serum, whereas "+" particles do combine, as would be expected. The extraordinary sensitivity of "+" particles to inhibitors in horse serum sets them apart from most of the other influenza viruses which are, like "−" particles, insensitive.

Electrophoresis of horse serum has revealed two major components with both hemagglutination-inhibiting and neutralizing activities: one migrates with α-globulins and the other with albumin. Thus, hemagglutination-inhibition is not due to one component and neutralization to another; rather, the inhibitory serum components possess both types of activity. Failure of normal horse serum to inhibit prototype viruses such as Lee or PR8 sets it apart from normal rabbit and human sera which inhibit not only "+" particles but many other influenza viruses as well.

These unusual features of inhibition of "+" particles by horse serum components raise the question whether the reactive sites on horse serum inhibitors are analogous to the sialic acid–containing reactive groupings of inhibitory components in other animal sera, of urinary mucoprotein and of erythrocytes. If it were postulated that the reactive sites on horse serum inhibitors are fundamentally different, *i.e.* did not contain sialic acid residues, it would also be necessary to postulate a unique reactive site on "+" virus particles. Yet all of the available evidence indicates that the reactive sites of "+" virus particles are analogous to the reactive sites of other influenza viruses for mucoprotein receptors. The outstanding feature of "+" particles is that they combine with extraordinary firmness with mucoprotein receptors for influenza virus.

Furthermore, in enzymatic experiments, "+" particles, purified neuraminidase, and *V. cholerae* filtrate all caused partial inactivation of the hemagglutina-

tion-inhibiting activity of horse serum, and inhibitory fractions, separated from horse serum by electrophoresis, were all susceptible to these agents. These results are significant in two ways; they indicate that virus receptor groupings of inhibitory components in horse serum probably contain neuraminic acid residues, and they also suggest that the receptors differ in some respects from those on rabbit serum components because the latter are much more susceptible to neuraminidase.

Indeed, the inhibitory activity remaining after treatment of horse serum with viral or soluble neuraminidase must be explained. The possibility that only one of the two major inhibitory components in horse serum possesses sialic acid–containing virus receptors appears to have been excluded by the finding that neuraminidase decreased the inhibitory activity of both of the major components to a similar degree.

Another possible explanation of the nature of virus receptors on horse serum inhibitors is that although all receptor groupings may contain neuraminic acid residues, not all of these residues are susceptible to the action of viral or soluble neuraminidase. This hypothesis obviates the necessity of postulating a new kind of a reactive site on the virus particle. Furthermore, Kuhn (20) has reported the isolation of a substance from cow colostrum which differs from neuramin-lactose, a substrate for neuraminidase, only by the presence of a linkage between neuraminic acid and the galactose residue at the 6 position, instead of the 3 position, as in neuramin-lactose. Neuraminidase fails to split neuraminic acid from this substance. If some such linkages were present in inhibitory mucoproteins of horse serum, the particles might attach to such a receptors, but not be able to free themselves by enzymatic action.

Whatever the correct explanation for the differences in reaction of virus with receptors may be, it is clear that the inhibitor-sensitive A2 virus particle can attach and form a relatively stable union at sites on the horse serum inhibitor at which neither the inhibitor-insensitive A2 particle nor the influenza viruses isolated prior to 1957 can attach.

Sensitivity of "+" Particles to Inhibitors in Normal Rabbit Serum.—In contrast to horse serum, normal rabbit serum inhibits hemagglutination by many influenza viruses isolated prior to 1957. In experiments with "+" and "−," influenza A2 virus particles, the former but not the latter were sensitive to rabbit serum inhibitors (1, 2). The complete insensitivity of the "−" particles sets them apart from most influenza viruses. The results obtained with electrophoretically separated fractions of normal rabbit serum and the "+" particles indicate that the same components possess both hemagglutination-inhibiting and neutralizing activities. The neutralizing activity can be taken as evidence that "+" particles form unusually strong bonds with inhibitors in rabbit serum. In contrast, many influenza viruses isolated prior to 1957 react with these inhibitors but form weaker bonds, *i.e.* hemagglutination is prevented

but infectivity is not neutralized by heated rabbit serum, and the influenza A2 "−" particles do not react at all.

Enzymatic treatment of rabbit serum with "+" particles caused a moderate reduction in the hemagglutination-inhibiting activity of serum as measured with "+" particles; treatment with "−" particles had no effect. The fact that treatment with "−" particles had no effect together with the fact that rabbit serum had no inhibiting effect on "−" particles, strongly suggests that "−" particles do not combine with inhibitors in rabbit serum.

V. cholerae filtrate and purified neuraminidase reduced markedly the inhibitory activity of rabbit serum for "+" particles. In the light of these findings there seems to be no reason to postulate that "+" particles react with receptors different from sialic acid–containing groupings with which many other influenza viruses react. Indeed, the combined evidence strongly favors the view that "+" particles react with sialic acid–containing receptors on inhibitory components in rabbit serum, but that for reasons which are not clear they form unusually firm bonds.

Thus it is clear that a single inhibitory substance such as urinary mucoprotein or a component of serum may cause different types of inhibition depending on the virus used to measure the activity. The difficulty in using a given virus as an indicator of the presence of a certain distinct type of inhibitor is apparent.

There is no direct evidence to explain the failure of "−" particles to react with inhibitory components in horse and rabbit sera. Clearly, "−" particles do possess reactive sites for sialic acid–containing receptor groupings on mucoproteins, because "−" particles are capable of reacting specifically with such groupings on urinary mucoprotein and erythrocytes. In addition, the inhibitory components in horse and rabbit sera appear to possess sialic acid–containing virus receptors. Therefore, to explain the failure of "−" particles to react with inhibitory components in horse and rabbit sera, it is necessary to postulate that steric hindrance prevents proper alignment of the reactive sites on virus and inhibitor.

In this connection results which have been obtained in studies with erythrocytes are pertinent. Myxoviruses are able to react with erythrocytes from mammalian, avian, amphibian, reptilian, and fish species (21, 22). It is likely that erythrocytes from species ranging from man to the dogfish possess sialic acid–containing receptor groupings for myxoviruses. However, within this wide species range of virus-erythrocyte interaction, instances can be found in which reaction does not take place. Structural fit as well as adsorptive forces may be involved in this phenomenon (22).

SUMMARY

Inhibitors present in normal human and animal sera prevented hemagglutination by and neutralized infectivity of inhibitor-sensitive influenza A2 virus.

Starch zone electrophoresis of sera indicated that the same serum components possess both hemagglutination-inhibiting and neutralizing activities. The greatest amount of inhibitory activity was found in normal horse serum, and the inhibitory activity increased with heating or treatment with concentrated solutions of urea. The inhibitory activities on human, ferret, and rabbit sera were markedly reduced but not completely eliminated by *V. cholerae* filtrate and purified neuraminidase. The inhibitory activity of horse serum was only moderately reduced by these agents. The nature of the horse serum inhibitor and the differences in the interactions of inhibitor-sensitive and insensitive influenza A2 virus particles and pre-1957 influenza viruses with receptors have been discussed.

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